Endogenous inhibitor of ecdysone synthesis in crabs

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Summary. Attempts to isolate the molt-inhibiting hormone (MIH) of crustaceans from crab eyestalks (ES) resulted in the characterization of xanthurenic acid as an inhibitor of ecdysone biosynthesis in the cultured Y-organ-complex (YOC) homogenate. It was also found that 3-hydroxy-L-kynurenine present in the ES is transformed into xanthurenic acid in the YOC and body fluid. Its mode of inhibitory action in ecdysone biosynthesis is probably inactivation of cytochrome P-450.
Key words. Molt-inhibiting hormone (MIH); inhibitor; ecdysone biosynthesis; crustaceans; xanthurenic acid.

During the early 20th century, biologists demonstrated[1, 2] that removal of eyestalks (ES) from crustaceans promotes molting and premature ecdysis, and that reimplantation of ES reverses this effect, thus suggesting the presence of a molt-inhibiting hormone (MIH)[3]. According to current views[4–7] a MIH produced in the X-organ of ES inhibits the release of ecdysone[8–11] from the Y-organ which is located in the thorax[4]. It has also been shown by in vitro experiments that crab tissues, especially the testis but not Y-organs, are capable of converting ecdysone to 20-hydroxyecdysone[12, 13], the circulating molting hormone[7, 8]. Despite many efforts, the chemical nature of MIH from ES remains to be clarified[14–17]. The purification and characterization of a neuropeptide, a putative MIH has recently been reported and the relationship between MIH and the hyperglycemic hormone (CHH) has been discussed[18]. The ecdysteroid titer in crabs is dependent on the molting stage[13, 19, 20], the season[21], locality, sex, maturity, etc., the interrelations of which are not clear, and it is this unreliability of assay animals that has been the main reason for the slow progress in MIH characterization.

We report here the chemical characterization of an endogenous inhibitor of ecdysone biosynthesis which is carried out by cultures of homogenized Y-organ and adhering tissues (Y-organ-complex or YOC). In conjunction with HPLC, the isolation was monitored by measuring the amount of ecdysone produced by the homogenized YOC culture with or without addition of fractionated ES extracts.

Materials, method and results. Callinectes sapidus (blue crab) and other crab species (a random mixture of sex, maturity, and molting stage) were collected off different coasts of USA and Japan. After immobilization of crabs by chilling on ice, their ES were uprooted from their bases with tweezers. The excised ES were frozen with dry-ice and lyophilized to prevent deterioration of the tissue for storage in the cold-room (~ 5 °C) as the MIH pool (see below). The destalked crabs were killed by acute freezing with liquid nitrogen and then stored below – 80 °C until used as the Y-organ source. Although the potency for ecdysone production undergoes slow deterioration during storage, about 90% of the animals thus treated were found to be usable (for three months) as the source of Y-organs; however, when the crabs were frozen after death instead of undergoing acute freezing while alive, about 40% of the Y-organs produced no ecdysone due to autolysis.

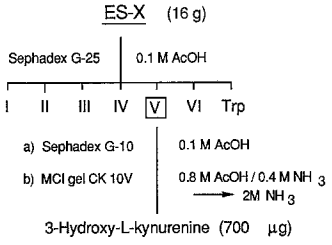
Ecdysone biosynthesis by YOC. The protocol for ecdysone biosynthesis using homogenized YOC culture is as follows. After thawing, the crab carapaces were removed by cutting the epimeral fracture lines, and the YOC was dissected. They were rinsed once with chilled 0.17 M KH_2PO_4/$NaHCO_3$ buffer, (pH 7.1, 1.0 ml/Y-organ) to remove the MIH accumulated on the tissues (see below), and homogenized in the chilled buffer (100 µl/Y-organ); this rinsing had a tendency to increase the ecdysone production by YOC. The suspension was transferred into centrifuge tubes (final volume 1.2 ml/Y-organ) and centrifuged below 5 °C at 7700 g for 20 min. The precipitate was centrifuged again with the same volume of buffer. To the combined supernatant containing the enzymatic system and its substrate, were added 0.2 mg streptomycin and 200 IU penicillin per 1 ml medium, and aliquots of 2.5 ml/Y-organ were incubated at 37 °C with shaking for 20 h; these conditions prevented organ decay and allowed the YOC homogenate to biosynthesize ecdysone. Upon termination of incubation, each aliquot was lyophilized and extracted with methanol. The extract was passed through SEP-Pak C 18 (Waters), washed with 5 ml of methanol and evaporated. The residue was submitted to quantitative analysis of ecdysone by HPLC using ERC-ODS-1161 (ERMA, Tokyo) (MeOH : CH_3CN : H_2O, 28 : 10 : 62, 247 nm). Ecdysone quantification was carried out by HPLC where 14,15-dehydro-20-hydroxyecdysone, prepared by dehydration of 20-hydroxyecdysone with 1% hydrochloric acid in methanol[22], was employed as an internal marker. The reproducibility of repeated runs of the same batch was within 6%.

The ecdysone thus detected in the cultured homogenates was usually 5 ~ 10 ng/Y-org, although in a few exceptional cases titers as high as a few hundred ng/Y-organ were encountered. The amount of 5 ~ 10 ng/organ was sufficient for assessment of the inhibitory effect since the limits of HPLC detection and quantification were 3 ng and 10 ng, respectively. The ecdysone produced in the culture medium was identified not only by HPLC but also by combined gas chromatography and mass spectrometry GL–MS of its trimethylsilyl derivative.

This procedure using combined YOC homogenates (e.g., prepared from 10 animals or 20 organs) rather than individual YOCs led to the leveling of variations among animals, and hence to a reduction in the number of control experiments. It has been noted that the ecdysteroid production is dependent on molting stage[13, 19, 20], season[21], etc. Although the relationship between the titers of ecdysone and its biosynthetic inhibitor is under investigation, the ecdysone titers are usually high in YOC cultures prepared from crabs collected in December–March and low in summer crabs.

Assay for the inhibitory action of ES. The ES stored as the MIH pool, 660 ES weighing 36 g, were extracted 6 times with 200 ml each of 0.1 M acetic acid, 100 °C for 10 min and centrifuged (1580 × g) for 15 min. The supernatant was lyophilized and the residual powder (16 g) was stored as the reference crude ES extract or ES-X (fig.). The inhibitory action of ES-X (in KH_2PO_4/$NaHCO_3$ pH 7.1 buffer) was assayed by co-incubation with the YOC homogenates for 20 h, 37 °C in an ES/Y-organ ratio of 1 : 1, and percent inhibition of ecdysone synthesis was calculated from the reduction in the ecdysone HPLC peak area as compared to that of the control containing no ES-X. The inhibitory activity manifested itself even when tested among different crab species; i.e., fiddler crab (*Geothelphusa dehaai*)/swimming crab (*Portunus trituberculatus*), swimming crab/blue crab (*Callinectes sapidus*), etc. The inhibitor(s) thus appear(s) to be species-nonspecific between ES donor and test crab. It was also found that the inhibitory activity in the ES-X was not destroyed upon heating at 100 °C for 30 min.

Isolation of 3-hydroxy-L-kynurenine. Attempts to isolate the MIH from the ES-X following the inhibitory assay led to the



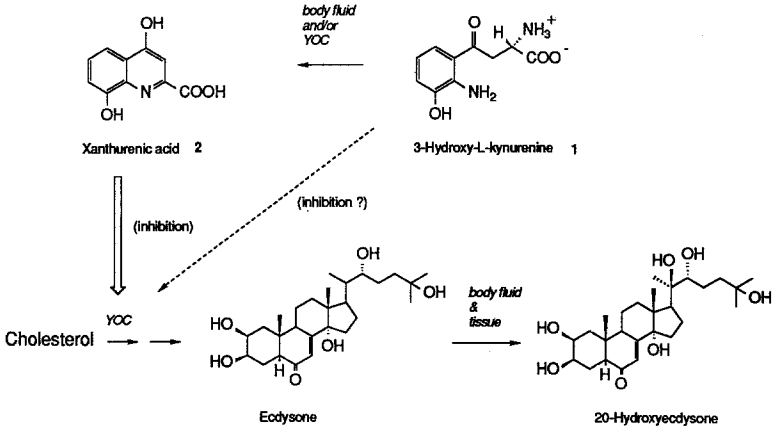
Isolation procedure of 3-hydroxy-L-kynurenine **1**.

characterization of 3-hydroxy-L-kynurenine (3-OH-K) (fig.). The 0.1 M acetic acid elution profile from the G-25 column was similar to those published earlier which followed an in vivo assay in the decapod (ES)/amphipod system[14]. Electrophoresis of fraction V on cellulose, 0.4 M acetic acid/0.12 M formic acid (1 : 1 v/v), pH 2.5, gave a pattern also closely resembling that reported previously[14]. Fraction V was further purified by Sephadex G-10, 0.1 M acetic acid, and cation exchange MCI gel, CK 10 V (Mitsubishi), 0.8 M acetic acid/0.4 M ~ 2 M ammonia, linear gradient elution, to yield 700 µg (ca. 1 µg/ES) of pure inhibitor, which was confirmed by co-culture with YOC homogenate. The structure was identical with 3-OH-L-Kyn **1** in all respects: ultraviolet absorption (UV), electron impact mass spectrum (EI-MS), circular dichroic spectrum (CD), nuclear magnetic resonance spectrum (NMR), electrophoresis and amino acid analysis. EI-MS m/z 322 (as diacetyl methylester); UV (in 0.005 M HCl) 224 nm (ε 18 400), 266 nm (ε 7600), 370 nm (ε 4000); CD (in 0.005 M HCl) 321 nm ($\Delta\varepsilon$ + 1.0), 273 nm ($\Delta\varepsilon$ + 0.5), 370 ($\Delta\varepsilon$ − 0.2). Incubation with authentic 3-OH-L-Kyn indeed exhibited inhibitory activity although the potency was less than that of the crude ES extract.

Isolation of xanthurenic acid. When xanthurenic acid **2** (scheme), the key metabolite of 3-OH-L-Kyn, was tested for MIH activity, surprisingly it was found to inhibit in vitro ecdysone synthesis more strongly than its precursor. An active search for **2** itself then led to its detection in ES-X. Thus ES-X was dissolved in aqueous ammonium acetate (0.17 M, 250 µl/ES, pH 5, a solvent system found to be efficient for xanthurenic acid extraction) and centrifuged at 1580 g for 15 min. The supernatant was placed on a Asahipak GS 320 column (Asahi Chem. Ind.) and treated with aqueous ammonium acetate to elute **1**, and then with 20% acetonitrile in 0.17 M ammonium acetate to elute **2** which was co-chromatographed with an authentic specimen on HPLC (GS 320) for its identification. The amounts of **1** and **2** as estimated from 920 ES were 3.4 µg and 2.0 µg/ES, respectively (from HPLC, 240 nm detection). The ED_{50} of ES-X, i.e., the effective dose of ES-X leading to a 50% inhibition of ecdysone synthesis was estimated to be ca. 1 mg ES-X, namely 136 ng of **1** and 78 ng of **2**. Even when assuming a 100% conversion of **1** → **2**, this combined amount of **1** and **2** appeared to account for most of but not the full potency of ES-X. It is unclear at this stage whether the inhibitory action of **1** is due to its transformation to **2** during incubation or whether it is an inherent inhibitor.

Detection of 3-hydroxy-L-kynurenine (1) and xanthurenic acid (2) from the X-organ-sinus gland complex[4] *and conversion of 1 → 2.* Parallel to the analytical-scale isolation of **1** and **2** from ES-X, eight X-organ/sinus gland complexes were carefully removed from fresh ES in ice-cold physiological saline, extracted with the ammonium acetate buffer, and centrifuged; HPLC indicated the amounts of **1** and **2** per X-organ to be 28 ng and 128 ng, or ~ 1% and ~ 7% of the amounts in ES-X, respectively. Transformation of **1** into **2** (scheme) was also demonstrated, using a crude enzyme preparation obtained from the crab body fluid in which a 3-h incubation at 37 °C led to a 60% conversion. For preparation of the enzyme, the body fluid extracted with 0.17 M $KH_2PO_4/NaHCO_3$ buffer was treated with a large excess of casein (to inhibit proteolysis) and further with saturated aqueous ammonium sulfate. After centrifugation, the residue was dialyzed for 24 h against water. The non-dialyzed fraction was filtered through Sephadex G-200 (Pharmacia) with the buffer and used as the crude enzyme preparation.

It is conceivable that **1** is transported from the X-organ to the Y-organ and converted into **2** in the body fluid and YOC; the YOC homogenate before incubation often contains certain amounts of **2** (ca. 350 ng) but little (ca. 35 ng) or nothing of **1**. Incubation of the YOC homogenate with authentic **1** resulted in a steep decrease (~ 90%) in the level of **1** but no rise in the level of **2**. This result suggested that the loss of **2** was caused by catabolism together with its consumption during the course of inhibitory action on ecdysone biosynthesis. Incubation of **2** (but not of **1**) with oxidized cytochrome C resulted in a typical binding ESR spectrum ($Fe^{III}-O$), which could not be reversed by NADPH-cytochrome C reductase; the substrate binding was also indicated by a shift of the Soret peak (γ) from 410 to 415 nm (details to be described elsewhere). It is conceivable that **2** is reacting in a similar manner with cytochrome P-450, preferably at the site



Inhibitors involved in ecdysone biosynthesis: 3-OH-L-K **1** produced in the ES is presumably converted into xanthurenic acid **2** in the body fluid and the YOC. Xanthurenic acid possibly interferes with the P-450-induced hydroxylations leading to ecdysone although the involvement of P-450 has as yet not been proven. The 20-hydroxylation of ecdysone was not inhibited by **2** upon co-incubation with the crab body fluid which transformed ecdysone into 20-hydroxyecdysone.

of iron porphyrin, thus leading to inactivation of cytochrome P-450 in ecdysteroid synthesis.

Discussion. Present studies have shown that the inhibitory action of ES-X on ecdysone biosynthesis is species-nonspecific among crabs; it was also found that the inhibitory action of **1** and **2** occurs at the site of the Y-organ. However, **2** does not suppress the 20-hydroxylation of ecdysone in the body fluid although we have shown the latter to contain an ecdysone-20-hydroxylase (by ecdysone → 20-hydroxyecdysone conversion, scheme). Experiments with insect tissues [23] and ecdysone 20-monooxygenase from insect sources [24] have also demonstrated that **1** and **2** do not inhibit the subsequent 20-hydroxylation of ecdysone. In addition, injection of **1** and **2** into lobsters [25] neither lowered the circulating ecdysteroids nor prolonged the molt cycle of eyestalk ablated lobsters. It is noteworthy that insects also release endogenous inhibitors of microsomal oxidations [26], though their biological function in vivo is unknown. Ongoing investigations include endocrinological studies of **1** and **2**, clarification of their modes of inhibitory action in ecdysone synthesis, identification of a further MIH-active fraction (if any) in ES-X, in vivo assays with crabs and lobsters, and clarification of the functional relationships between the recently isolated neuropeptide [18] and **1** and **2**.

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Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males

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Summary. Protamines were extracted from the sperm of fertile and infertile human males and the relative proportion of protamines 1, 2, and 3 were determined by scanning microdensitometry following electrophoresis of total protamine in polyacrylamide gels. The proportion of the three protamines was found to be similar in sperm obtained from different normal males. The distribution of protamines in sperm obtained from a select group of infertile males producing an elevated level of large sperm heads, in contrast, was different from that of the fertile males.
Key words. Human sperm; human protamine; protamine 1; protamine 2; protamine 3; infertility; gene expression.

Numerous studies have demonstrated that the DNA of human sperm is packed into the sperm nucleus by three different protamine molecules [1–7]. As in other placental mammals [2, 8–11], human protamines are synthesized and deposited onto DNA late in spermiogenesis, replacing the majority of the somatic histones and other transition proteins. This repackaging of DNA appears to 'deprogram' the bulk (85–90%) of the male genome and condense the DNA into a highly compact, biochemically inert nucleoprotamine complex. Only a small proportion (10–15%) of the DNA sequences remain packaged by a special group of sperm specific histones [12].

Each of the three human protamines has recently been characterized by several different laboratories [4–7, 13]. The primary sequence of protamine 1 (HP 1) was found to be similar to sequences obtained for bull [14], boar [15], ram [16] and mouse [17] protamine 1. The amino terminal sequence of each protamine 1 begins with the conserved sequence ala-arg-tyr-arg-cys-cys, the proteins contain precisely 50 amino acids, and the bulk of the arginine residues are concentrated in the